CHAPTER 3

RESULTS

1. Expression of WT1 protein in human cancer cell lines

The expression levels for WT1 protein were determined in human cell lines: MCF-7 (breast cancer cell), MDA-MB-468 (breast cancer cell), PC3 (prostate cancer cell) and LNCaP (prostate cancer cell). The result showed that WT1 expressed at high levels in two bands, 52 and 54 kDa, in MDA-MB-468 and LNCaP whereas only one band, 54 kDa, found in MCF-7 and PC3 (Fig. 4).

2. siRNA system for knockdown of WT1 has been developed

To determine the feasibility of creating a microenvironment in which WT1 can be selectively depleted, the siRNAs against WT1 ($siRNA_{WT1}$) were synthesized by Invitrogen, the sequence were complementation to three different regions of WT1 mRNA, namely, $siRNA_{WT1}$ R88, $siRNA_{WT1}$ R89 and $siRNA_{WT1}$ R90. We then tested these $siRNA_{WT1}$ for their ability to knock down expression of target proteins. MCF-7 cells were transfected with each $siRNA_{WT1}$ by using 200 and 400 nM concentration at 48 and 72 h. Western blot analysis was performed to ensure that $siRNA_{WT1}$ could specifically inhibit WT1 protein expression. The result showed that WT1 protein expression was clearly 70% decreased at 200 nM of $siRNA_{WT1}$ R88 and 90% for $siRNA_{WT1}$ R90 at 72 h (Fig. 5A and 5C), but $siRNA_{WT1}$ R89 has no effect on WT1 protein expression (Fig. 5B). Furthermore, we found that mixed siRNA (R88, R89 and R90) at 200 and 400 nM final concentrations was sufficient to reduce WT1 protein expression at 48 h compared with the control (Fig. 5D). In addition, $siRNA_{WT1}$ did not cause a nonspecific downregulation of gene expression, as determined by the β-actin control.

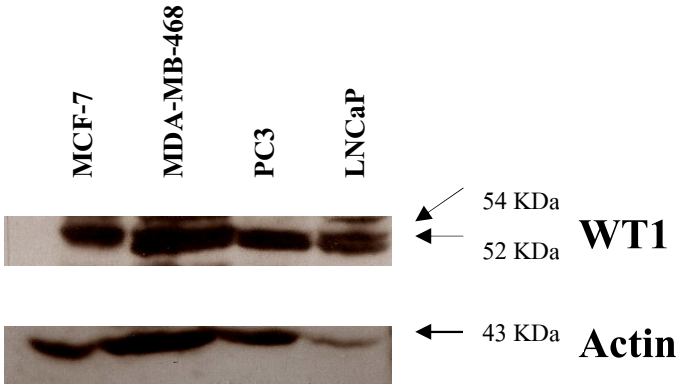
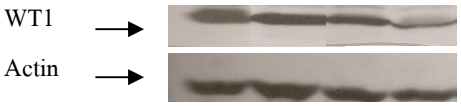


Figure 4. Expression of WT1 protein in human cancer cell lines. WT1 protein expression was analyzed by Western Immunoblotting. Fifty microgram cell lysates were loaded onto 12% SDS–PAGE. WT1 protien was detected in all of these breast and prostate cancer cell lines studied.

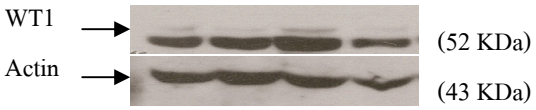
A.

Region	88			
Dose (nM)	control		200	
Time (h)	48	72	48	72



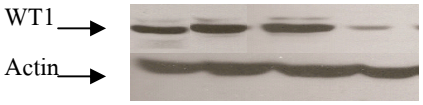
B.

Region	89			
Dose (nM)	control		200	
Time (h)	48	72	48	72



C.

Region	88			
Dose (nM)	control		200	
Time (h)	48	72	48	72



D.

Region	Mixed				
Dose (nM)	control		200		400
Time (h)	48	72	48	72	72

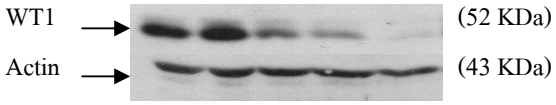


Figure 5. siRNA system for knockdown of WT1 has been developed. Fifty microgram cell lysates were separated by 12% SDS-PAGE and immunoblotted with antibody against WT1. The effect of the dose of siRNA and time on the level of WT1 protien expression was demonstrated. Expression levels were normalized for a loading by probing for β-actin. (A= $siRNA_{WT1}$R88, B= $siRNA_{WT1}$R89, C= $siRNA_{WT1}$ R90, D= Mixed $siRNA_{WT1}$).

$siRNA_{WT1}$ inhibited WT1 protein expression and the growth of MCF-7 breast cancer cell in dose - dependent manner

To determine the effects of $siRNA_{WT1}$ on cells proliferation, MCF-7 cells were transfected with mixed $siRNA_{WT1}$ at 0, 25, 50, 100, 200, 400 and 800 nM final concentrations for 72 h. The control cells were added with only transfection agent. After $siRNA_{WT}$ transfection, the number of viable cells were evaluated by trypan blue dye exclusion assay at final concentration 0.2% and counted with hematocytometer. We found that 25 nM mixed $siRNA_{WT1}$ was sufficient to inhibit the proliferation of MCF-7 cells (71% inhibition). In addition, the number of cells was also decreased in dose-dependent manner until reached the plateau phase at 200 nM. The degrees of growth inhibition as compared with control cells were 80%, 85%, 93%, 95%, 95% at 50, 100, 200, 400, 800 nM siRNA respectively (Fig. 6). The pictures of MCF-7 cells growing in cell culture plates, captered under phase contrast microscopy after trasfected with siRNA are shown in figure 7**.** These results confirmed that $siRNA_{WT1}$ could inhibit cancer cell proliferation in dose-dependent manner. Furthermore, the levels of WT1 protein in MCF-7 cells were measured by Western immunoblotting at these corresponding time points (Fig. 8). This study showed that the levels of WT1 protein were also decreased in dose- dependent manner. The silencing effect of $siRNA_{WT1}$ on WT1 protein expression was significantly observed at 50 nM concentration while WT1 protein was completely abolished at the dose of 800 nM based on this detection method.

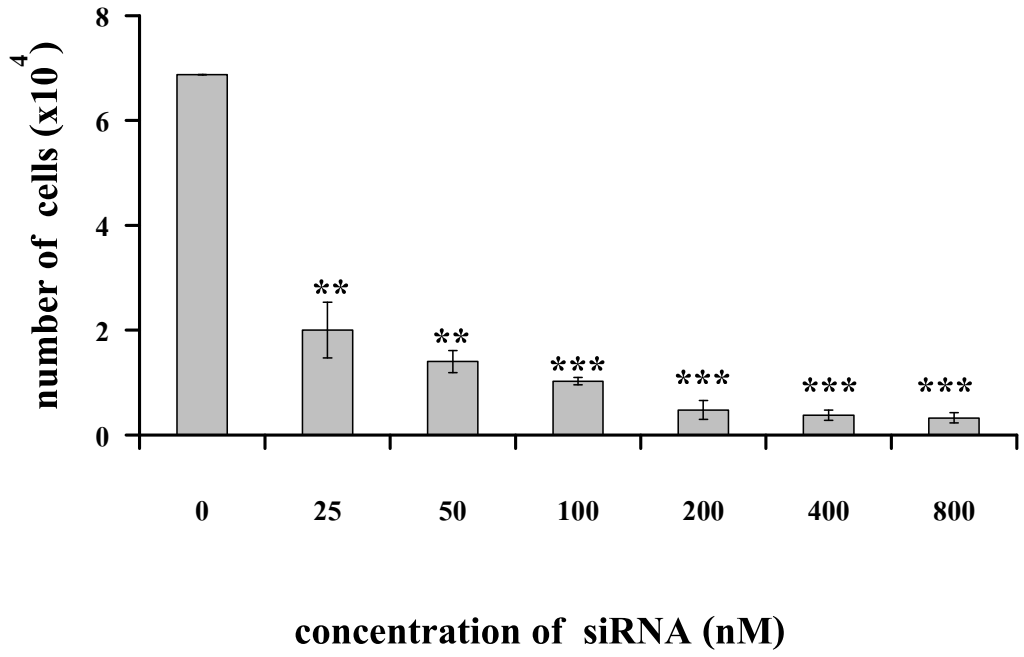


Figure 6. Mixed $siRNA_{WT}$ **led to a reduced cellular proliferation in dose -dependent manner.** MCF-7 cells were transfected with different concentrations of $siRNA_{WT1}$ with 0, 25, 50, 100, 200, 400 and 800 nM for 72 h. The cells were stained with trypan blue at final concentration 0.2% and the unstained survival cells were counted with hemacytometer. The data represented the average value for three independent transfection experiments, each was performed in triplicate. Significant growth inhibition was first observed at 25 nM mixed siRNA in which more than 70% growth inhibition was demonstrated (*, $p< 0.05$ by two-sample t test, comparing with 0 nM as a control cells). The greater degree of growth inhibition was observed in the higher concentrations of siRNA.

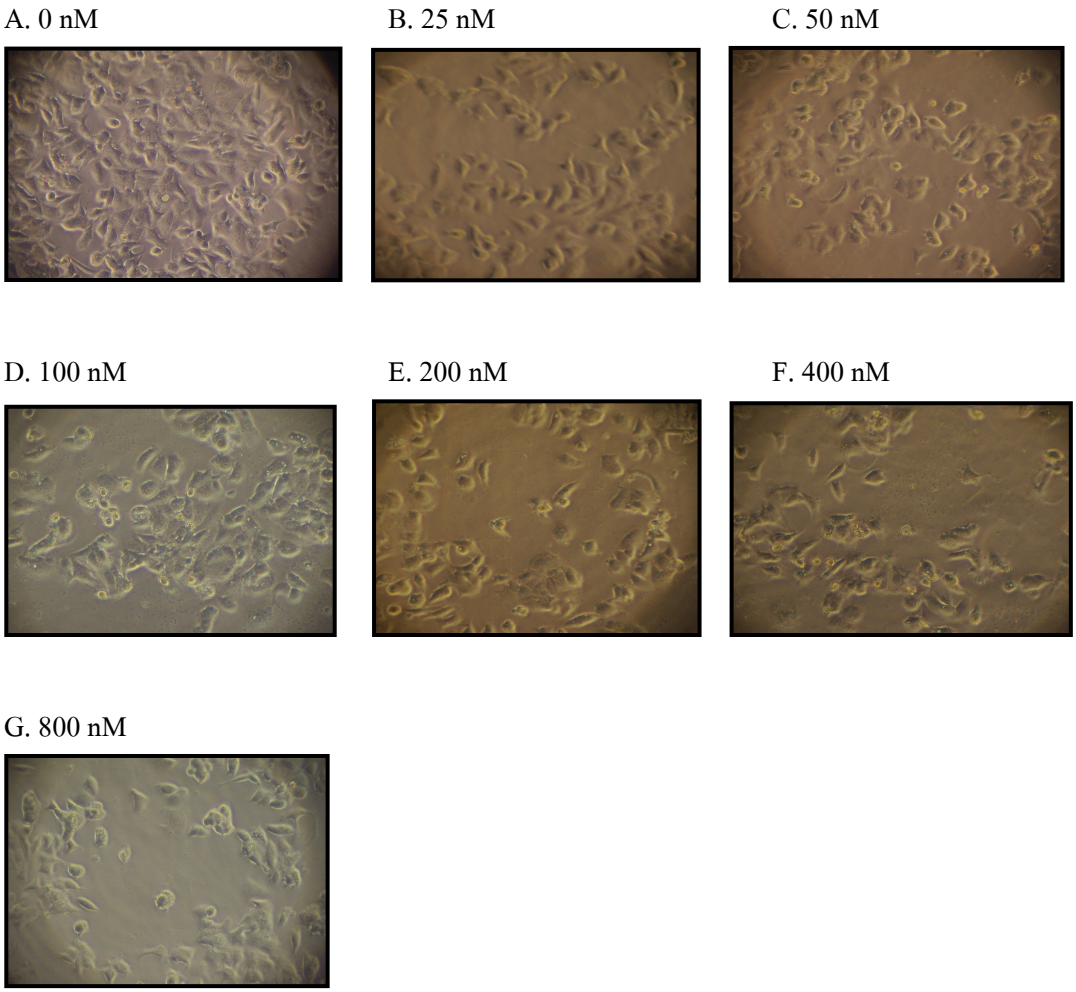


Figure 7. Reduction in number of MCF-7 cells by siRNA$_{WT1}$ was dose - dependent. MCF-7 cells were transfected with 0, 25, 50,100, 200, 400 and 800 nM siRNA$_{WT1}$ for 72 h. *A-G*, cells were observed under Olympus phase contrast microscope (10X). This finding confirmed the dose-dependent manner of siRNA$_{WT1}$ effect on cell proliferation.

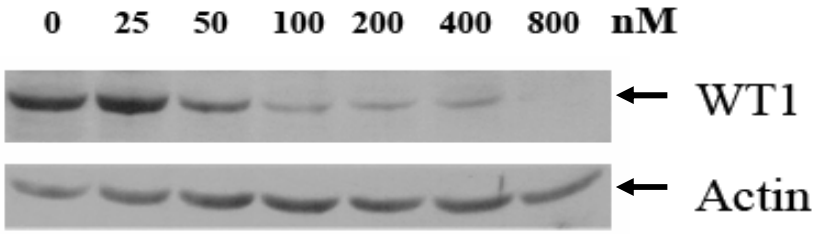


Figure 8. siRNA effectively knocked down WT1 protein expression in MCF-7 in dose-dependent manner. MCF-7 cells were transfected with different concentrations of $siRNA_{WT1}$. At 72 h after transfection, 50 μg of total protein extracted from the cell was subjected to western immunoblotting to detect WT1 and actin. $siRNA_{WT1}$ concentraiton which could induce the significant WT1 inhibition was 100 nM. At 800 nM siRNA, WT1 protein was completely abolished.

Silencing of WT1 protein expression and growth inhibition in MCF-7 breast cancer cells by $siRNA_{WT}$ in a time - course experiment

To determine the effects of $siRNA_{WT1}$ on the proliferation in MCF-7 cells during experimental time course, the cells were transfected with 100 nM mixed $siRNA_{WT1}$ or only transfection agent (as a control). The number of viable cells were then measured by trypan blue exclusion assay and counted under hemacytometer. Our data showed that $siRNA_{WT1}$ inhibited the growth of MCF-7 cells in time-dependent (Fig. 9). The significant growth inhibitory effect of $siRNA_{WT1}$ as compared with control cell was first observed at 24 h after $siRNA_{WT1}$ transfection (44% inhibition) and reached the maximum effect (92% growth inhibition) at 120 h after transfection. The pictures of MCF-7 cell growing in culture after transfected with $siRNA_{WT1}$ at different time points, captured under phase contrast microscopy are shown in figure 10. This finding confirmed the time-dependent growth inhibitory effect of $siRNA_{WT1}$ on the breast cancer cells. To confirm that this effect was attributable to WT1 downregulation, the levels of WT1 protein were also monitored by western immunoblotting along this experimental time course (Fig. 11a and 11b). This data showed that $siRNA_{WT1}$ induced the decrease in the levels of WT1 protein expression in time-dependent manner. At this 100 nM concentration of $siRNA_{WT1}$, the silencing effect on WT1 protein expression was first observed at 24 h after transfection and increased along the experimental time course until reached the maximum effect at 120 h after transfection, when no WT1 protein signal was detected by this method.

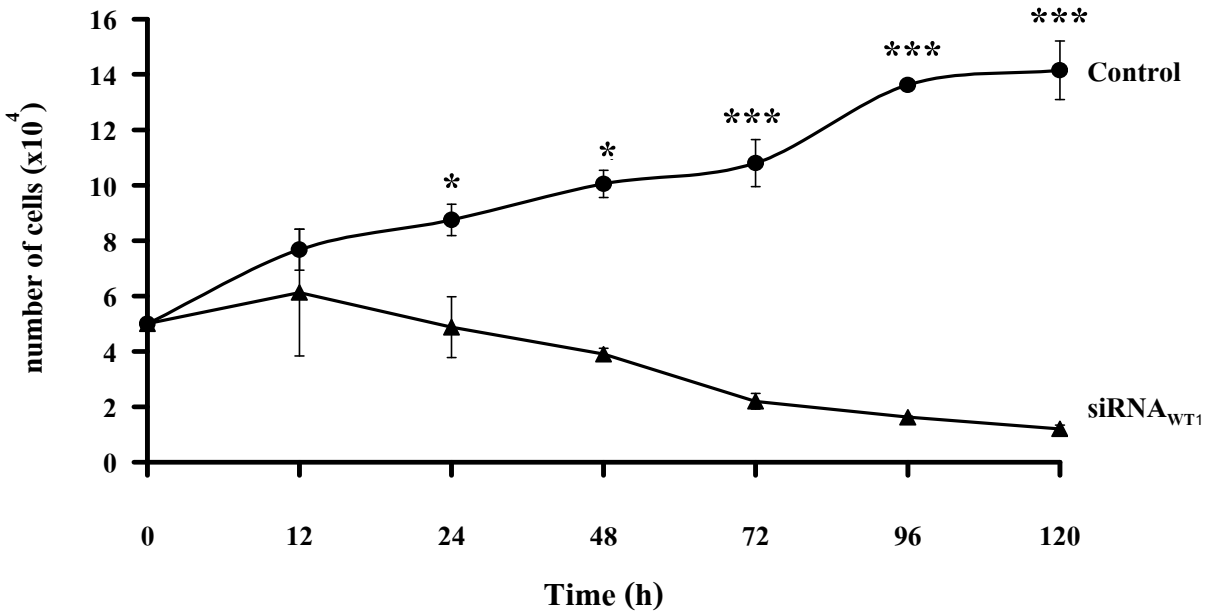


Figure 9. Mixed siRNA$_{WT}$ led to a reduced cellular proliferation in time-dependent manner. MCF-7 cells were transfected with 100 nM siRNA$_{WT1}$ for 12, 24, 48, 72, 96, and 120 h. The cell were stained with trypan blue at 0.2% final concentration and counted with hematocytometer. The data represented the average value from three independent transfection experiments, each was performed in triplicate. Significant growth inhibition was first observed at 24 h after transfection (44% inhibition) and reached the maximum level at 120 h (92% inhibition) (***= $p < 0.005$; *= $p<0.05$ by two-sample t test, comparing with control).

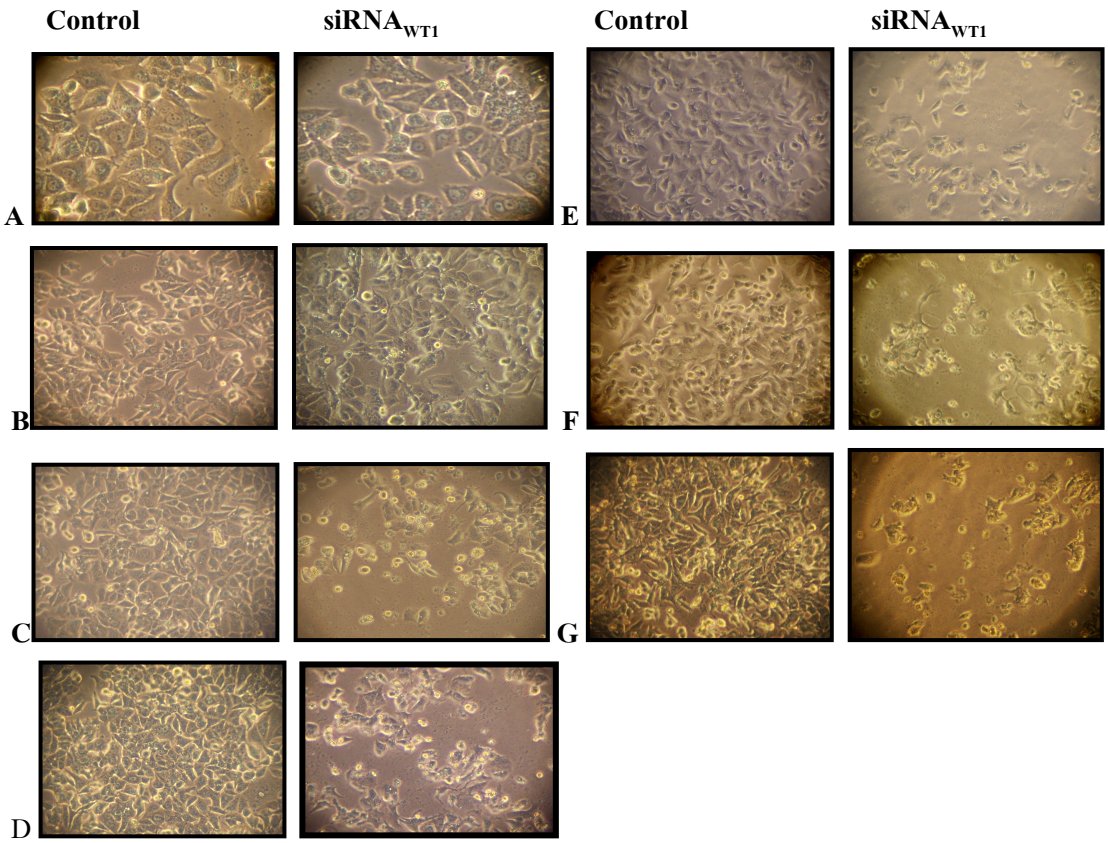
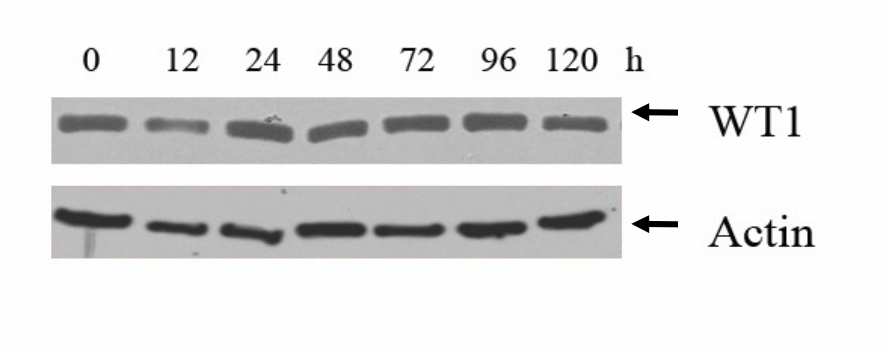


Figure 10. **Reduction in numbers of MCF-7 cells by siRNA$_{WT1}$.** MCF-7 cells were transfected with 100 nM siRNA$_{WT1}$ or control for 12, 24, 48, 72, 96 and 120 h. A= 0 h, B= 12 h, C= 24 h, D= 48 h, E= 72 h, F= 96 h, and G= 120 h. Cells were observed under Olympus phase contrast microscope, (20X in 0 h and 10X in 12, 24, 48, 72, 96, 120 h).

A.



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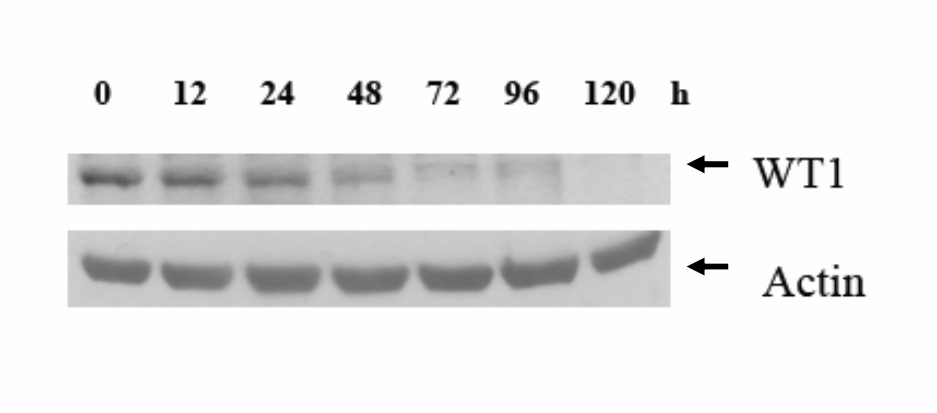

Figure 11. Decrease in WT1 protein level in breast cancer cells by siRNA in time - course experiment. MCF-7 cells were transfected with 100 nM of $siRNA_{WT1}$ or control. At 12, 24, 48, 72, 96 and 120 h after transfection, total protein was extracted and subjected to western immunoblotting for WT1 protein detection. At this concentration, significant decrease in WT1 protein was first observed at 24 h and reached the maximum level at 120 h after transfection. A= contol cells, B= siRNA-transfected cells.